

# Domain IVa of laminin $\alpha 5$ chain is cell-adhesive and binds $\beta 1$ and $\alpha V\beta 3$ integrins through Arg-Gly-Asp

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**Abstract** The globular domain IVa from the short arm region of mouse laminin  $\alpha 5$  chain was obtained by recombinant production and shown to be a cell-adhesive substrate and to bind  $\alpha V\beta 3$  integrin in solid-phase assays. These interactions were blocked by RGD peptides and a restricted panel of anti-integrin antibodies. The two RGD sequences present in  $\alpha 5$ IVa were shown by site-directed mutagenesis to make different contributions to cell adhesion but were equivalent in binding  $\alpha V\beta 3$  integrin. A quantitative radioimmuno-inhibition assay was established based on domain  $\alpha 5$ IVa which demonstrated distinct amounts of  $\alpha 5$  chain in various tissues, particularly in vessel walls. There it could play a role in angiogenesis steps requiring RGD-dependent integrins. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Basement membrane; Cell–matrix interaction; Radioimmunoassay; Recombinant protein

## 1. Introduction

The laminins represent a large protein family and are involved in basement membrane assembly and major cell–matrix interactions. They are composed of disulfide-bonded  $\alpha$ ,  $\beta$ ,  $\gamma$  chains and 14 isoforms of these chains have been extensively or partially characterized [1,2]. The largest variant known so far is the  $\alpha 5$  chain with about 3700 residues [3] which is present in laminin-10 ( $\alpha 5\beta 1\gamma 1$ ) and laminin-11 ( $\alpha 5\beta 2\gamma 1$ ) [4–7]. These laminins are already expressed during early somite stages of mouse development [8] and can be detected in many tissues during further development and also in the adult stage [4,7,9–11]. An important function of laminin  $\alpha 5$  chain is also strongly indicated from gene targeting in mice which die at embryonic days 14–17 with massive failures in neural tube closure, digit septation and glomerulogenesis [8,12].

A major function impaired in the  $\alpha 5$  chain knock-out mice could be integrin-mediated cell adhesion which, as shown from studies with laminin-10 and -11, involves the  $\alpha 3\beta 1$  and  $\alpha 6\beta 1$  integrins [5,6,13]. This implicated, in analogy to other laminin isoforms [1,2], binding of the receptors to the C-terminal LG modules of the  $\alpha 5$  chain. A cell-adhesive pepsinized form of laminin-10/11, however, did not recognize these integrins [14]. Additional cellular interactions could include heparin binding epitopes as shown for C-terminal and N-terminal

recombinant fragments of the  $\alpha 5$  chain [15,16] and the lutheran blood group glycoprotein [17,18]. A further possible interaction site could be one or both of the two RGD sequences present in domain IVa within the short arm of the laminin  $\alpha 5$  chain [3]. Such RGD recognition sequences are frequently found in other extracellular proteins including fibronectin and vitronectin and have the ability to bind to various integrins [19]. RGD sequences are rarely found in laminins and only a cryptic binding site has so far been identified in the mouse laminin  $\alpha 1$  chain [20,21].

In the present study we have examined these possibilities by recombinant production of domain IVa which folded into a globular structure. This fragment promoted cell adhesion mediated by RGD-dependent integrins with different contributions made by the two RGD sequences as shown by site-directed mutagenesis.

## 2. Materials and methods

### 2.1. Sources of proteins, antibodies and cells

Laminin-1 in complex with nidogen-1 was obtained from a mouse tumor basement membrane [22] and vitronectin was purified from human plasma [23]. Synthetic peptides were prepared by our internal facilities (Dr. L. Moroder) and characterized by mass spectrometry. Inhibitory monoclonal antibodies against human  $\beta 1$  (A1B2),  $\alpha 5$  (BIIG2) and  $\alpha 6$  (GoH3) integrin subunits were kindly supplied by Drs. C. Damsky and A. Sonnenberg. Other inhibitory monoclonal antibodies were obtained from commercial sources (Dianova, Hamburg; Biomol) and are identified in Table 1. The sources of cell lines used have been previously identified [20].

### 2.2. Production and characterization of recombinant protein domains

An expression vector for mouse laminin domain  $\alpha 5$ IVa was amplified by RT-PCR using RNA from embryonic endothelial cells [24] (kindly supplied by Antonis Hatzopoulos) as template and the 5' primer GTCAACTAGTTACCCGCTGCTTCTGTTTCGGG and 3' primer GTCACCTCGAGCTAGGGGACACATCGGCCTAGGAAG. After restriction with *SpeI/XhoI* the cDNA was ligated into the episomal expression vector pCEP-Pu containing the BM-40 signal peptide [25]. Mutations were introduced by fusion PCR [26]. These vectors were used to transfect human kidney 293-EBNA cells and to collect serum-free conditioned medium [25]. For numbering of sequence positions [3] we added 83 more residues to the N-terminus (data bank accession number AJ293593) including the  $\alpha 5$  chain signal peptide.

Serum-free medium (1 l) was passed over a DEAE cellulose column (2.5 × 8 cm) equilibrated in 0.05 M Tris–HCl, pH 8.6 followed by elution with a linear 0–0.4 M NaCl gradient (400/400 ml). The  $\alpha 5$ IVa fragments and mutants eluted around 0.2 M NaCl and were obtained in purified form by molecular sieve chromatography on a Superose 12 column (HR 16/50, Pharmacia) equilibrated in 0.2 M ammonium acetate, pH 8.6. Production and purification of the mouse laminin  $\alpha 1$  chain fragment  $\alpha 1$ IVa has been previously described [21].

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Electrophoresis in SDS gels, Edman degradation on a 473 sequencer (Applied Biosystems) and amino acid analysis for determining protein and hexosamine concentrations followed established protocols. Rotary shadowing was used prior to electron microscopy [27].

### 2.3. Cell adhesion and integrin binding assays

Cell adhesion to coated micro-well plates, staining of adherent cells with crystal violet and colorimetry have been described [28]. Inhibitors were used over a 100-fold concentration range and added to the cells prior to seeding to the coated wells. Integrin  $\alpha V\beta 3$  was purified by affinity chromatography from human placenta and used as immobilized ligand in solid-phase binding assays [29].

### 2.4. Immunological assays

Immunization of rabbits, affinity purification of antibodies, ELISA and radioimmunoassays followed standard protocols. Adult mouse tissue extracts were prepared with neutral buffer containing 10 mM EDTA followed by the same buffer containing detergents [30] and used in the radioimmuno-inhibition assay specific for  $\alpha 5IVa$ . These extracts were also analyzed by inhibition assays specific for laminin fragments  $\alpha 1VI/V$  [31] and a similar assay for  $\alpha 2VI/V$  (T. Sasaki, R. Giltay, U. Talts, R. Timpl and J.F. Talts, submitted). All these extracts were examined in duplicate using three different dilutions which showed at the most a variability not exceeding 20–30% of the mean. Indirect immunofluorescence staining with affinity-purified antibodies (5  $\mu\text{g/ml}$ ) was performed on cryostat sections of adult mouse tissues [32]. Control incubations with normal rabbit IgG gave negative results.

## 3. Results

Domain  $\alpha 5IVa$  corresponds to position 1631–1863 of mouse laminin  $\alpha 5$  chain and was produced by human 293-EBNA cells transfected with an episomal expression vector [25]. These cells secreted the recombinant fragment  $\alpha 5IVa$  in reasonable amounts (4–7  $\mu\text{g/ml/day}$ ) into serum-free culture medium which was used for purification by conventional chromatography. The purified fragment showed a single 31 kDa band in SDS gel electrophoresis (Fig. 1A) and a single N-terminal sequence APLVTR(C)F(C)F with the first four residues being derived from the signal peptide cleavage region of the vector. Rotary shadowing electron microscopy showed small globular structures (Fig. 1B) indicating the proper folding of fragment  $\alpha 5IVa$ .

Fragment  $\alpha 5IVa$  corresponds to an L4 module type of laminins with the disulfide bond connectivities Cys1–3, Cys2–4, Cys5–6 and Cys7–8 and a 200 residue long globular insert in loop b between Cys2 and 4 [20,21]. It contains two potential

Table 1  
Inhibition of cell adhesion to laminin domain  $\alpha 5IVa$  by monoclonal antibodies to integrins and synthetic peptides

Inhibitors	HT1080 cells	A375 cells
Antibodies	Maximal inhibition in %	
Anti- $\beta 1$ (AIIIB2)	100	55
Anti- $\alpha 3$ (PIB5)	30	0
Anti- $\alpha 6$ (GoH3)	0	0
Anti- $\alpha V$ (AMF7)	0	30
Anti- $\alpha V\beta 5$ (PIF6)	80	40
Anti- $\alpha 5$ (BIIG2)	nt	70
Peptides	Maximal inhibition in % and IC <sub>50</sub> ( $\mu\text{M}$ )	
GRGDS	90 (3)	100 (4)
RGES	0 (> 500)	0 (> 500)
ETQRGDI	90 (6)	100 (10)
ANYRGDS	90 (15)	100 (10)

Inhibitions were examined by dose–response profiles starting with antibody dilutions strongly inhibiting cell adhesion in other adhesion assays and with 500  $\mu\text{M}$  peptides. IC<sub>50</sub> refers to concentrations required for half-maximal inhibition. nt = not tested.

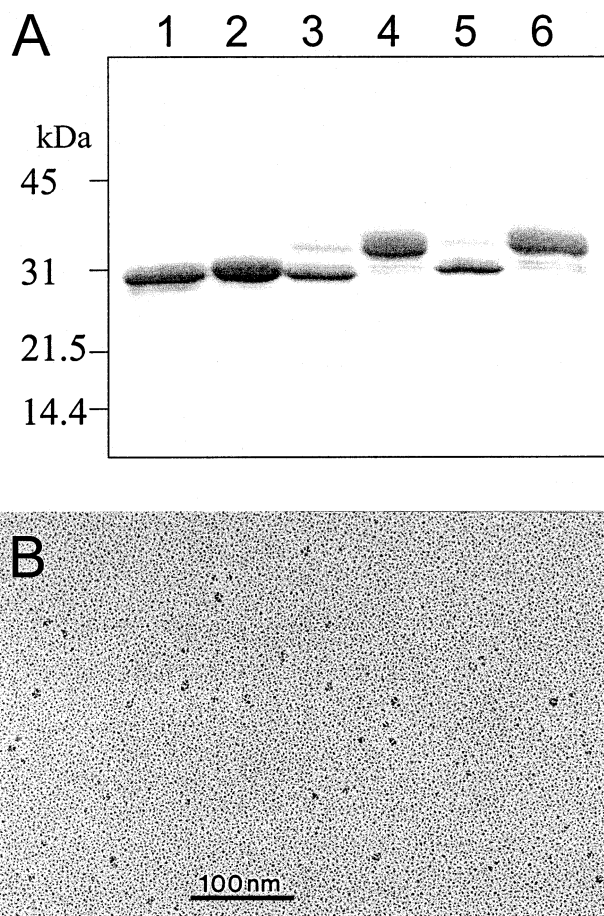


Fig. 1. Characterization of recombinant fragment  $\alpha 5IVa$  and mutants by SDS gel electrophoresis (A) and rotary shadowing electron microscopy (B). Lanes in A were loaded with wild-type  $\alpha 5IVa$  (lane 1), mutant R1723P (2), mutant R1839T (3,4) and the double mutant R1723P/R1839T (5,6). The latter two mutants were examined after separating their 31 and 34 kDa bands by molecular sieve chromatography. Wild-type  $\alpha 5IVa$  is shown in B.

cell binding RGD sites [17] within the sequences 1718-HSETQRGDIF in the center of loop b and 1834-CPA-NYRGDSC between Cys5 and 6 [3]. These RGD sites are changed to PGD and TGD, respectively, in the analogous domain IVa of laminin  $\alpha 3B$  chain [33] and we used these substitutions to produce the  $\alpha 5IVa$  mutants R1723P and R1839T and the double mutant R1723P/R1839T. These mutants were purified and showed the same 31 kDa band as  $\alpha 5IVa$  (R1723P) and in addition a 34 kDa band for R1839T and the double mutant (Fig. 1A). The increase in molecular mass was due to the generation of an *N*-glycosylation site (1837-NYT) as shown by a content of three glucosamines not found in the wild-type. All three mutants were indistinguishable from wild-type  $\alpha 5IVa$  when examined by ELISA with an antiserum against  $\alpha 5IVa$  (data not shown).

The activity of these RGD sites was initially examined in cell adhesion assays as shown for B16F10 melanoma cells (Fig. 2A) and revealed a distinct dose–response profile with half-maximal attachment for  $\alpha 5IVa$  at coating concentrations of 1–2  $\mu\text{g/ml}$ . Since a native laminin-10 was not available, a comparison was made with laminin-1 which showed a similar activity but with higher plateau levels (Fig. 2A). Similar dose–response profiles for both substrates were also observed with

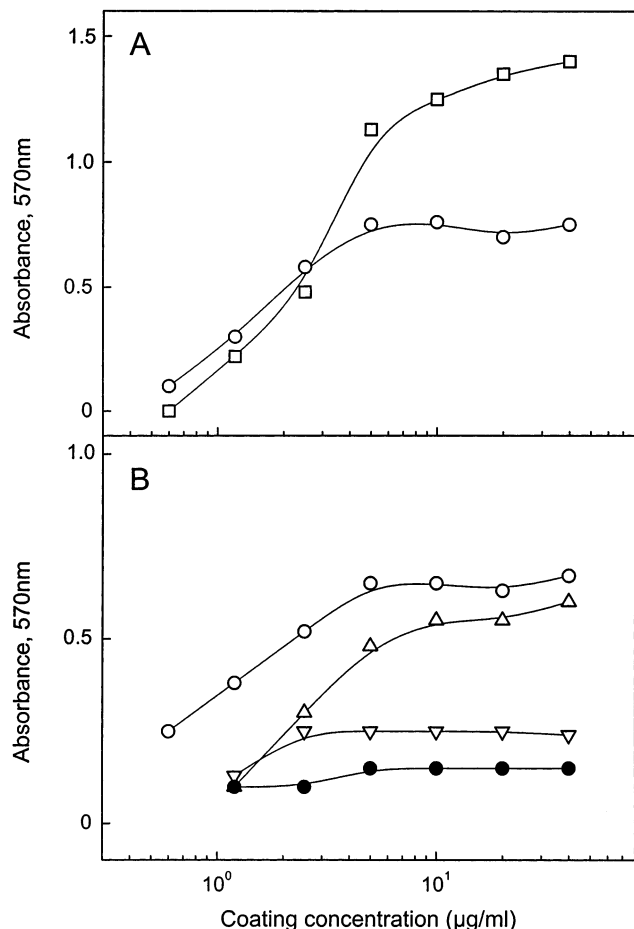


Fig. 2. Adhesion profiles of mouse melanoma B16F10 (A) and human melanoma A375 cells (B) to fragment  $\alpha 5IVa$  and  $\alpha 5IVa$  mutants. Wells were coated with  $\alpha 5IVa$  (○), mutants R1723P (▽) and R1839T (△), the double mutant R1723P/R1839T (●) and laminin-1 (□).

human fibrosarcoma HT-1080 and melanoma A375 cells and rat schwannoma RN22 cells. However, several other cell lines such as human A431, Saos-2 and HBL-100 and rat Rugli glioma failed to adhere to the  $\alpha 5IVa$  substrate.

The specificity of interactions was further studied in inhibition assays with monoclonal antibodies against human integrins and with synthetic RGD peptides (Table 1). This demonstrated moderate to complete inhibition by anti- $\beta 1$  antibodies and variable levels of inhibition with antibodies against  $\alpha V\beta 5$  integrin and  $\alpha V$  or  $\alpha 5$  subunits. However, antibodies against  $\alpha 3$  and  $\alpha 6$  integrin subunits showed either no or only a marginal effect. A more uniform 90–100% inhibition, which was half-maximal at 3–15  $\mu M$  peptide, was achieved with GRGDS, ETQRGDI and ANYRGDS, the latter two corresponding to authentic  $\alpha 5IVa$  sequences. This inhibition was specific since no effect was observed with 100-fold higher concentrations of synthetic RGDs (Table 1).

The relative contributions of the two RGD sites to cell adhesion were further studied by the  $\alpha 5IVa$  mutants described above. This showed for A375 cells (Fig. 2B) a distinct reduction in the activity of mutant R1723P which was further augmented in the double mutant. The loss of activity was distinctly less in the mutant R1839T where a five-fold increase in the coating concentration was necessary to obtain a compa-

table reaction as with  $\alpha 5IVa$ . Similar profiles as shown in Fig. 2B were observed with three more cell lines. In a second test we used purified RGD-dependent  $\alpha V\beta 3$  integrin [29] in solid-phase binding assays (Fig. 3). This showed for  $\alpha 5IVa$  a distinct binding profile with half-maximal binding achieved at 0.5 nM and was thus comparable to the binding of vitronectin (0.3 nM). The two mutants showed a 10–20-fold lower activity while no binding was observed with the double mutant up to a concentration of 50 nM.

A broad distribution of laminins containing the  $\alpha 5$  chain during developmental and adult stages was previously shown by analyzing mRNA expression and antibody staining of tissues [4,7–11]. Because quantitative immunological assays were not available, a more precise correlation to the tissue contents of other laminin  $\alpha$  chains was impossible. We have now generated a rabbit antiserum against  $\alpha 5IVa$  which showed a good titer (1:2500) in ELISA and no cross-reaction with the related laminin  $\alpha 1IVa$  chain fragment at 1:50 dilution. This high specificity was confirmed by a radioimmuno-inhibition assay for  $\alpha 5IVa$  which showed inhibition by  $\alpha 5IVa$  in a dose-dependent manner with 0.2 nM  $\alpha 5IVa$  required for 50% inhibition. No inhibition was observed with 1000-fold higher concentrations of  $\alpha 1IVa$  and several more unrelated laminin fragments (data not shown).

The radioimmunoassay was also inhibited by EDTA and detergent extracts of adult mouse tissues which showed the same dose-response profile as the  $\alpha 5IVa$  reference inhibitor. This set the stage for a quantitative evaluation which showed  $\alpha 5$  chain contents in the range of 3–159 pmol/g wet tissue (Table 2). This was 3–78% of the concentration of either  $\alpha 1$  or  $\alpha 2$  chain that dominate a particular tissue. The relative contents of  $\alpha 5$  chain was, however, low in heart and skeletal muscle which contain large amounts of  $\alpha 2$  but only negligible amounts of  $\alpha 1$  chain. Affinity-purified antibodies against  $\alpha 5IVa$  were also examined by immunofluorescence staining (examples shown in Fig. 4) and showed a localization to many basement membranes in kidney, lung and skin and to a large variety of small and larger vessels. A restricted staining of vessels was, however, observed in heart and skeletal muscle

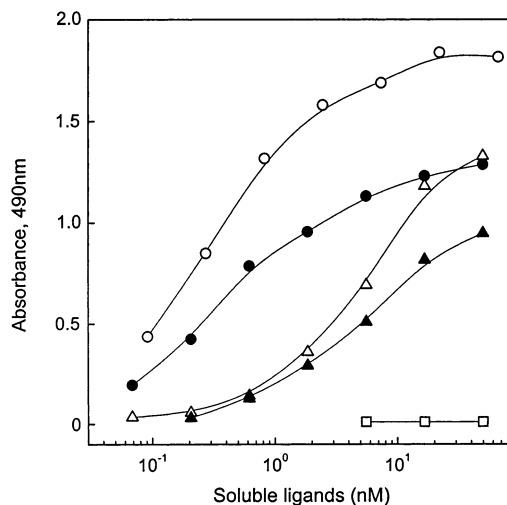


Fig. 3. Solid-phase binding assay to immobilized  $\alpha V\beta 3$  integrin. Soluble ligands used were vitronectin (○), laminin fragment  $\alpha 5IVa$  (●) and its mutants R1723P (△), R1839T (▲) and R1723P/R1839T (□).

in agreement with a relatively low content of  $\alpha 5$  chain in these tissues (Table 2).

#### 4. Discussion

The successful recombinant production of laminin fragment  $\alpha 5IVa$  demonstrated as shown before for  $\alpha 1IVa$  [21] that laminin L4 type modules represent an autonomous unit capable of folding into a globular structure. Domain  $\alpha 5IVa$  was also shown to be a cell-adhesive substrate as anticipated from the presence of two RGD sequences [3]. These interactions can be mediated by several RGD-dependent integrins including  $\alpha 5\beta 1$ ,  $\alpha V\beta 3$ ,  $\alpha V\beta 5$  and possibly  $\alpha V\beta 1$  as shown by inhibition and direct binding assays. Since both RGD sequences are conserved in the human laminin  $\alpha 5$  chain (K. Tryggvason, personal communication) it is likely that they will have a similar cell-adhesive activity which may be conserved among most mammalian species. This conservation does not exist for the *Drosophila* laminin  $\alpha$  chain which corresponds to  $\alpha 5$  [34]. There is also no conservation in the mouse laminin  $\alpha 3B$  chain [33] which, as shown here for the  $\alpha 5$  double mutant, will abolish cell adhesiveness and  $\alpha V\beta 3$  integrin binding. Thus it appears that the laminin  $\alpha 5$  chain is unique among all other laminin chain isoforms known so far in binding to RGD-dependent integrins.

Depending on the assay used, the activity of both RGD sequences in domain  $\alpha 5IVa$  did not seem to be always equivalent. There was no significant difference in the binding to purified  $\alpha V\beta 3$  integrin while cell adhesion seemed to be stronger with the 1723-RGD than the 1839-RGD site. This could reflect the topological positions of both sites being either in the center of the globular b loop or constrained in the short c loop between a disulfide bridge which may have a different impact on the binding of soluble versus cell membrane-bound integrins. Yet, it could also be due to different affinities of individual integrins which are more complex in the cell adhesion assay and also include a substantial fraction of  $\beta 1$  integrins.

The only other cell-adhesive RGD site of laminins identified so far has been located to the mouse  $\alpha 1$  chain but pepsin digestion is necessary to generate an active fragment P1 [20]. This fragment was adhesive for all four cell lines binding to  $\alpha 5IVa$  but also for cells which did not adhere to  $\alpha 5IVa$  indicating a different repertoire of integrins involved in binding.

Table 2  
Contents of laminin  $\alpha 5$  chain in EDTA/detergent extracts of adult mouse tissues

Tissue	$\alpha 5IVa$ assay	$\alpha 1VI/V$ assay	$\alpha 2VI/V$ assay
Placenta	159	484	10
Heart	23	1	237
Skeletal muscle	3	< 1	140
Lung	21	2	27
Kidney	28	78	21
Thymus	6	2	16
Skin	4	3	11

Contents (pmol/g wet tissue) were determined by radioimmuno-inhibition assay specific for  $\alpha 5IVa$  and were compared to the contents of laminin  $\alpha 1$  and  $\alpha 2$  chains determined recently (T. Sasaki, R. Gil-tay, U. Talts, R. Timpl and J.F. Talts, submitted) by similar assays with recombinant  $\alpha 1VI/V$  and  $\alpha 2VI/V$ . The values represented the sum of EDTA and detergent extracts, which, as shown previously, solubilize 71–92% of the  $\alpha 1$  and  $\alpha 2$  chains.

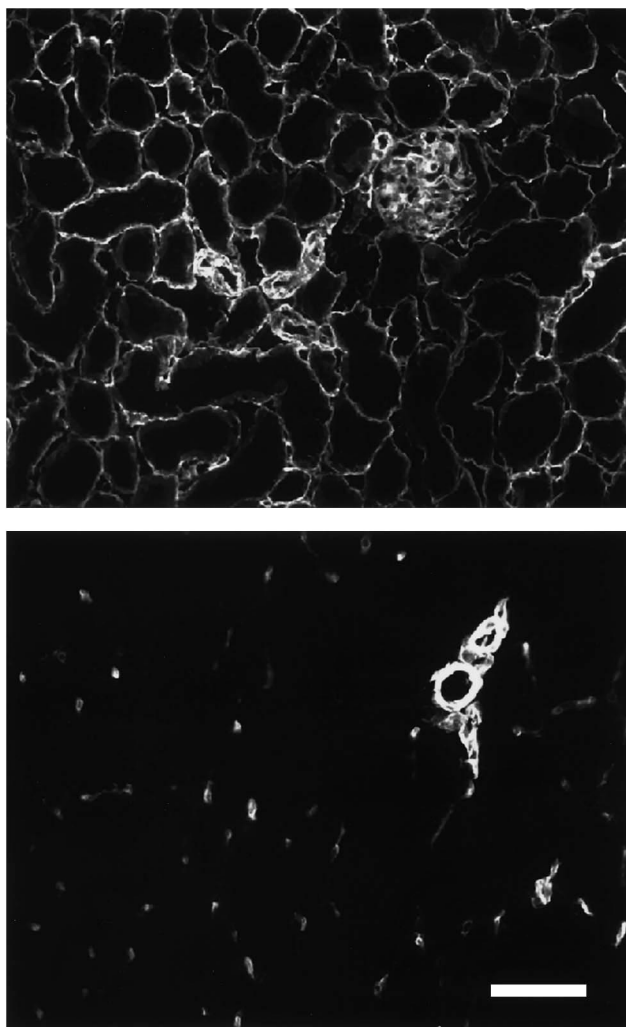


Fig. 4. Indirect immunofluorescence staining with affinity-purified antibodies against  $\alpha 5IVa$  of kidney (top) and skeletal muscle (bottom) from adult mice. Bar: 50  $\mu m$ .

Furthermore, the binding of purified  $\alpha V\beta 3$  integrin to laminin P1 was much weaker than to vitronectin [29] unlike the binding to  $\alpha 5IVa$  observed in this report. The RGD in the  $\alpha 1$  chain is located in a small disulfide-bonded loop next to domain  $\alpha 1IVa$  but does not seem to be very active in cell adhesion and  $\alpha V\beta 3$  integrin binding as shown with a corresponding recombinant fragment indicating that it is still masked by the globular domain  $\alpha 1IVa$  [21]. It is furthermore not known whether proteolytic activation of the RGD site of laminin-1 occurs in tissues and this RGD is also not conserved in the human laminin  $\alpha 1$  chain. The biological significance of this cell adhesion remains therefore questionable.

The RGD-mediated cell adhesion to  $\alpha 5IVa$  was apparently as strong as to laminin-1 which is mainly mediated by  $\alpha 6\beta 1$  and some other integrins not involved in RGD recognition [19]. Cell adhesion to laminin-10/11, however, is about 10-fold stronger and can be inhibited by antibodies against  $\beta 1$ ,  $\alpha 3$  and  $\alpha 6$  integrin subunits [5,6,13]. This strongly indicated that other domains presumably derived from the C-terminal region of the  $\alpha 5$  chain provide stronger cell-adhesive epitopes than  $\alpha 5IVa$ . As discussed below, it seems likely that recognition of  $\alpha 5IVa$  by other integrins may be mainly required for mediating other activities such as cell migration.

Antibodies against domain  $\alpha 5IVa$  were used for the quantitation of  $\alpha 5$  chains in adult mouse tissue extracts and demonstrated particularly high concentrations in placenta, kidney, heart and lung amounting to 10–80% of the levels of  $\alpha 1$  or  $\alpha 2$  chain. This agreed with a strong  $\alpha 5$  mRNA expression in the same tissues exceeding distinctly those of  $\alpha 1$  and  $\alpha 2$  mRNA [4]. Such large differences were, however, not detected by the radioimmunoassay indicating distinct differences in the translation efficiency or stability of mRNAs encoding different laminin  $\alpha$  chains. Immunohistology with the  $\alpha 5IVa$  antibody confirmed previous data [4,7,9–11] by showing a distinct staining of various renal and alveolar basement membranes and of capillary and larger vessel walls. In heart and muscle staining was restricted to the vessels which together with the radioimmunoassay data suggested a particularly high  $\alpha 5$  chain content in these structures.

The strong deposition of  $\alpha 5$ -containing laminins in vessel walls together with the ability to bind RGD-dependent integrins suggests a role for such interactions during cell migration, angiogenesis and the final conversion into a stable, non-leaky neovasculature [35]. Similar activities were already shown for  $\alpha V\beta 3$  and  $\alpha V\beta 5$  integrins by inhibition studies with monoclonal antibodies [36–38]. The extracellular ligands involved have so far not been identified and could include laminins-10/11, as shown for  $\alpha 5$  knock-out mice which fail to vascularize renal glomeruli [12]. Interestingly, one of the inhibiting antibodies [37] was also shown here to block substantially  $\alpha 5IVa$ -mediated cell adhesion (P1F6, see Table 1). Further studies will, however, be required to show that adhesion-blocking antibodies against  $\alpha 5IVa$  produce a similar inhibition of angiogenesis.

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